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Preparative and analytical purification of DNA from agarose

(NaI/acetone precipitation/DNA–glass complexes/molecular hybridization)

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ABSTRACT **Two procedures were developed for removing DNA from agarose after electrophoretic separation of DNA fragments according to size. Both involve dissolving the DNA-containing agarose in NaI. The preparative technique uses binding of DNA to glass in the presence of NaI. The method is rapid and convenient, and DNA of all molecular weight ranges can be recovered in high yield and without degradation. The DNA is free of agarose and remains susceptible to digestion by restriction enzymes. The analytical technique uses selective precipitation of DNA with acetone and has been adapted to molecular hybridization scans of sequences in agarose gels. The sequence-monitoring system is quantitative, directly measuring the proportion of the probe complementary to a given DNA fragment and vice versa. It is especially suitable for analyzing restriction enzyme digests of DNA in mapping experiments.**

The use of agarose gels for separating DNA of various size classes has blossomed with the advent of restriction endonucleases (1, 2). However, the purification of DNA fragments from the agarose matrix for further study has long been problematic. Although several techniques have been devised for this purpose (3–12), none has satisfactorily eliminated the problems of incomplete separation of agarose from DNA, degradation or other modifications of DNA (including the loss of "restrictability"), low yield, inconvenience, etc. (13). The problems are often magnified when large preparative gels are used or when multiple samples are analyzed.

We report here two simple techniques for separating DNA from agarose. Both techniques involve as a first step the solubilization of agarose in the chaiotropic salt, NaI. The second step is either binding of the DNA to glass or selective precipitation with acetone. The techniques are used jointly for some work; the acetone precipitation technique to locate specific fragments of DNA in resolving gels and to measure the proportion of the probe complementary to each fragment, and the glass-binding method to recover the fragments for further study.

MATERIALS AND METHODS

Restriction endonucleases were purchased from Bethesda Research Laboratories (Bethesda, MD). Agarose (grade II) was purchased from Sigma. Low-temperature agarose was a gift from Bio-Rad. Viral DNA was from Bethesda Research Laboratories. *Escherichia coli* DNA was from P-L Biochemicals. *E. coli* [^{14}C]DNA was a gift from D. Strayer. DNA was prepared from human spleen as described (14). ^{125}I-Labeled RNA, a gift from W. Prensky (15), was purified by chromatography on CF11 columns and stored under ethanol.

A saturated solution of NaI, refractive index of 1.5000, was made according to D. Strayer (personal communication). NaI (4, lb., 1.8 kg) was dissolved in 995 ml of warm water, filtered through nitrocellulose membranes, and allowed to stand at room temperature until crystals were formed. Sodium sulfite was added to saturation as an antioxidant.

The best studied glass powder was flint glass prepared from ground scintillation vials (American Flint Glass Co.). Glass particles were separated according to size by sedimentation through water at unit gravity. "Large" particles sedimented faster than 6 cm/min, "medium" particles sedimented between 1 and 6 cm/min, and "powder" sedimented more slowly than 0.25 cm/min. Silica gel and porous glass beads were unsuitable for DNA purification (see *Results*).

Methods I: Analytical Electrophoresis and Removal of DNA from Agarose. Vertical slab gels (3 mm depth) of 0.3–3.0% agarose were cast in a Bio-Rad model 220 support. For small numbers of samples and agarose gels of 0.6% or greater, combs were used to form the sample wells, usually 20 mm wide. For low-percentage agarose gels, posts of agarose were fixed to the top of the slab with melted 0.3% agarose. To eliminate "trailing" of the DNA at the edge of the slot, we applied DNA samples to the slab gels in the following manner. The sample wells were nearly filled with 5 mM NaOAc/2 mM EDTA/40 mM Tris acetate, pH 7.8. The DNA sample was held at 45°C for 10 min, then mixed with glycerol and melted low-temperature agarose to 2–10% and 0.2% final concentrations (wt/vol), respectively. When the sample gelled, the buffer was withdrawn and the well was filled with low-temperature agarose at 45°C. Electrophoresis was at 20 V for 15–30 hr. DNA was visualized by staining with ethidium bromide. Each slot was cut into 50–100 slices of 1.2 mm with a parallel razor blade assembly.

For analytical removal of DNA from agarose gel slices, the slices were placed in heavy-wall, 10 × 75 mm glass tubes (Pyrex no. 9820). Double- or single-stranded, sonicated (14) DNA from *E. coli* (50 μg) was added to each slice and the volume was brought to 0.8 ml with saturated NaI. The ratio of saturated NaI to aqueous solution from any other source (e.g., gel slice, carrier DNA) exceeded 2:1. The agarose was dissolved by gently rocking the suspension for several hours at room temperature or by vigorously mixing on a Vortex mixer for a few seconds. Acetone was added to 33% (0.4 ml), the contents were thoroughly mixed on a Vortex mixer, and the diffuse DNA precipitate was allowed to form at room temperature for 60 min. Evaporation was minimized by covering the rack of tubes with a large sheet of Parafilm. The tubes were centrifuged for about $10^4 \times g$-hr at 15°C. The supernatants were decanted and the tubes were washed four times with 75% acetone in water. Large numbers of samples were processed in the RC3 Sorvall and J6 Beckman centrifuges, by using 12-mm adaptors with retaining pads.

The tubes were dried under reduced pressure for 15–20 min. A slight NaI residue at the lip was not harmful. The clear pre-

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Abbreviations: rDNA, DNA complementary to rRNA; C_0t, DNA concentration in moles of nucleotide per liter multiplied by time in sec.

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cipitate was dissolved in 10–25 μl of 70% (vol/vol) formamide/0.2 M potassium phosphate at pH 6.8 containing labeled probe. Formamide/phosphate buffer was spread over the entire DNA precipitate by gentle tapping; the tube was then overlaid with about 200 μl of mineral oil. The tubes were covered with no. 4 red caps (Federal Scientific, Bethesda, MD).

The tubes were placed at 90–100°C for 10 min, then incubated at hybridization temperature for the desired length of time. RNA·DNA hybridization proceeded without DNA reannealing in formamide/phosphate buffer at 44°C (16, 17). The hybridization reaction was terminated by direct addition to the tube of 1 ml of 0.45 M NaCl/0.045 M Na citrate containing 20 μg of RNase A (Sigma) per ml and 50 units of RNase T1 (Calbiochem) per ml. The solutions were mixed, then incubated for 2 hr at 37°C. The samples were chilled and brought to 10% with trichloroacetic acid. The precipitate was collected on type GF/C glass fiber filters (Whatman). Background levels were under 0.5% of the input radioactivity. The mineral oil largely passed through the filter. ^{125}I was measured in a gamma counter. To determine the radioactivity of the internal markers, we shook the filters overnight with 1 ml of H_2O/NCS tissue solubilizer (Amersham) (0.15:1, vol/vol) then counted them in 10 ml of toluene-based scintillant containing 0.1 ml of glacial acetic acid.

Methods II: Preparative Electrophoresis and Removal of DNA From Agarose. A 5-inch (12.7-cm) diameter gel system designed by David Strayer was used (unpublished data). Preparative gels of 0.4–0.6% agarose gave high resolution of fragments from $0.53 \cdot 10^6$ to $33 \cdot 10^6$ daltons, starting from 12 mg of DNA digested with restriction enzymes having six base-pair-recognition sites. Slices from preparative gels were weighed and dissolved in 2 ml of saturated NaI per g of agarose, usually in screw-cap, polypropylene, centrifuge tubes (no. 2089, Falcon). Radioactive DNA was added to monitor recovery. At least 1 mg of glass powder was added per μg of DNA expected to be in the agarose slice. The tube was revolved slowly end-over-end at 25°C until all the DNA was bound to the glass. The rate of binding of DNA to glass was proportional to the concentration of glass surface; Fig. 1*B* can be used to calculate binding rates.

Glass powder was collected by centrifugation at the lowest permissible *g*-force. DNA–glass clumps sedimented quantitatively at less than 5000 × *g* for 5 min. DNA–glass was then washed with about 100 vol of 70% (wt/vol) NaI. To do this without degradation of high molecular weight DNA, we suspended the particles gently as large aggregates and revolved them for 30 min at 25°C, allowing residual agarose to diffuse into the NaI. DNA–glass was again collected by centrifugation. Particles were washed gently with the largest convenient volume of a mixture of 50% buffer (20 mM Tris·HCl, pH 7.2/0.2 M NaCl/2 mM EDTA) and 50% ethanol to remove NaI.

The DNA–glass was suspended in about 10 vol of 20 mM Tris·HCl, pH 7.2/0.2 M NaCl/2 mM EDTA. DNA was eluted at 37°C for 30 min with occasional gentle mixing. Glass was removed by centrifugation at 10,000 × *g* for 20 min. DNA was precipitated from ethanol and dissolved in 5 mM NaOAc/2 mM EDTA/40 mM Tris acetate, pH 7.8.

RESULTS

Binding of DNA to Glass. Both DNA and agarose are soluble in high concentrations of NaI; and in NaI, glass binds DNA. The two major classes of glass tested, soda lime and borosilicate, bound DNA with comparable effectiveness (Fig. 1*A*). Small amounts of DNA can be bound to continuous glass surfaces; the interior surface of a 10 × 75 mm tube holds about 1 μg of DNA; that of a scintillation vial, about 10 μg. Glass fiber filters bind

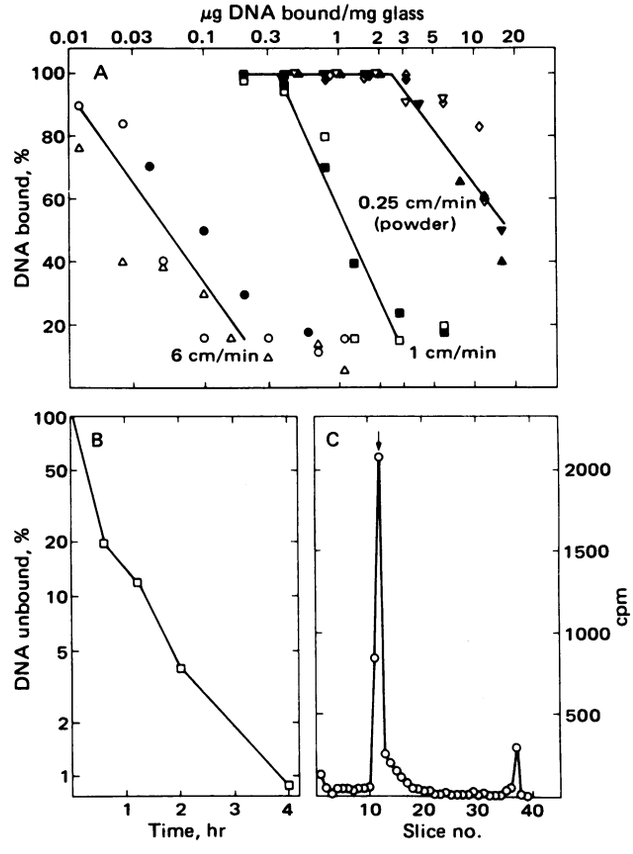

FIG. 1. Binding of DNA to glass. (*A*) Capacity of glass. DNA was mixed with 10 mg of glass particles in 0.25 ml of 5 mM NaOAc/2 mM EDTA/40 mM Tris acetate, pH 7.8. Saturated NaI (0.6 ml) with or without 10 mg of agarose was added and DNA was allowed to bind to the glass for 15 min at 25°C with mixing. Glass was separated from the solution by centrifugation. △, Large flint glass particles; ○ and ●, large borosilicate glass particles; □ and ■, medium flint glass particles; ◇, ▼, and ▲, flint glass powder; ♦ and ▽, silica powder. ●, ♦, ■, and ▼, Plus agarose; remainder, no agarose. Sizes of glass are defined in *Materials and Methods*. (*B*) Kinetics of binding. Saturated NaI (32 ml) was used to dissolve 16 g of 0.6% agarose in a 50-ml Falcon polypropylene centrifuge tube. *E. coli* [^{14}C]DNA (10 μg) and silica powder (50 mg) were added and the tube was revolved end-over-end at about 6 rpm. At intervals, 1 ml was removed and the percent of the ^{14}C bound was determined. (*C*) Size of recovered DNA. [^3H]DNA from bacteriophage λ was run into a 0.3% agarose slab. The DNA was located after staining with ethidium bromide, then removed from agarose by binding to flint glass powder. The DNA was precipitated from ethanol, then rerun on a 0.3% agarose gel. This gel was sliced and the radioactivity of each slice was measured in a scintillation counter. Arrow marks the position of marker bacteriophage λ DNA.

DNA in NaI, but quantitative recovery in small volumes is difficult.

Although the binding of DNA to tubes or vials is an uncommonly easy way of separating DNA from agarose, the capacity is too small for most preparative experiments. The amount of DNA bound per weight of glass was increased dramatically by using glass powder (Fig. 1*A*). Moreover, the kinetics of binding of DNA to glass was improved with the powder (Fig. 1*B*). Porous glass beads bind extraordinary amounts of DNA, but the binding is slow and not quantitative and DNA degradation results during elution of the DNA from the pores. Silicic acid also has a very high capacity for DNA, but is difficult to handle mechanically and presents similar recovery problems.

The glass-binding technique is uniquely suited for the rapid removal of high molecular weight DNA from agarose. DNA

from bacteriophage λ is about 48,000 base pairs long and can be recovered undegraded (Fig. 1C). On the other hand, DNA fragments under 100 base pairs long were also recovered in high yield (not shown). DNA recovered from agarose by the glass-binding technique was a suitable substrate for restriction enzymes (see later). It was also infectious (P. Markham, D. Wheeler, and D. Strayer, personal communication).

Monitoring DNA Sequences in Agarose Gel Slices. DNA can be selectively precipitated from NaI with acetone or ethanol concentrations (33%) that do not precipitate agarose. As little as 0.4 vol of acetone caused DNA to precipitate, while about 1 vol was necessary to precipitate agarose (Fig. 2A). Essentially no NaI was precipitated, but traces of agarose coprecipitated with DNA. The rapidity of DNA isolation and the concentrating nature of the precipitation process are especially useful in hybridization experiments.

Though small amounts of DNA in NaI can be precipitated from acetone and recovered by centrifugation, it quickly became apparent that the most convenient form of the method would require the addition of carrier DNA to the test DNA. Carrier DNA provided a visible precipitate to follow, minimized the binding of the test DNA to the walls of the centrifuge tubes, and allowed complete sedimentation in a 15- to 30-min, low-speed centrifugation. Fig. 2B shows that DNA of widely variant molecular weights are quantitatively precipitated with this procedure. The DNA precipitate includes NaI, which lowers the t_m (midpoint of thermal transition) of DNA and could be expected to interfere with molecular hybridization. NaI was removed by several washes with 75% acetone.

The molecular hybridization system was constructed simply by adding a few microliters of a labeled probe in appropriate buffer and overlaying the liquid with mineral oil. The rate of hybridization of RNA to DNA recovered from agarose was equivalent to hybridization under standard conditions (Fig. 2C). The rate of hybridization was not appreciably affected by increased chain lengths over 1000 nucleotides. Finally, in high

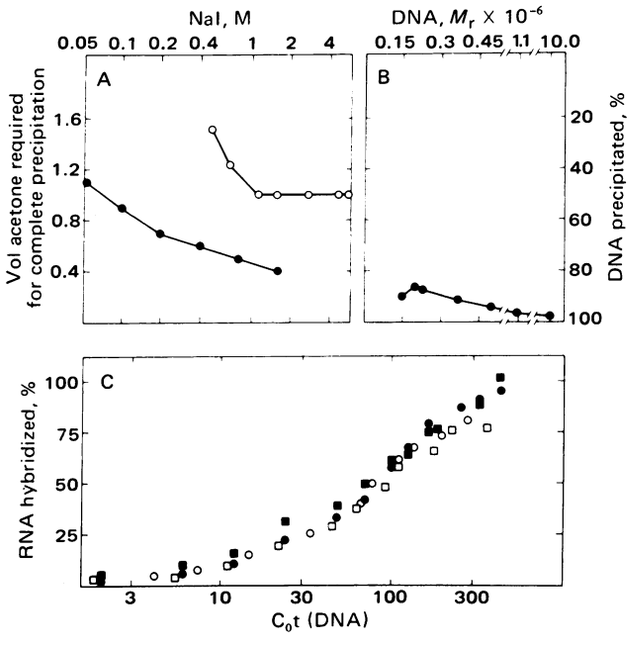

FIG. 2. Precipitation of DNA from acetone. (A) Effect of NaI concentration. Calf thymus DNA (0.25 mg/ml) (●) or agarose (1.2–6 mg/ml) (○) was dissolved in various concentrations of NaI. The smallest amount of acetone required to form a stable, visible precipitate was determined. Similar curves were obtained for ethanol. (B) Effect of DNA chain length. [^{3}H]DNA from simian virus 40 digested with *Hae* III or [^{14}C]DNA from *E. coli* was electrophoresed through 1% agarose. Slices containing specific DNA fragments were cut from the gel and dissolved in NaI in the presence of 50 μg of carrier DNA; then DNA was precipitated from acetone. DNA precipitates were dissolved in 10% acetic acid and reduced with a drop of 2-mercaptoethanol. Radioactivity was measured in Aquasol. (C) Kinetics of hybridization of acetone-precipitated DNA. Human DNA (50 μg) was added to tubes containing 250 μl of 1% agarose (or water) dissolved in 550 μl of saturated NaI. The DNA was precipitated from acetone and the precipitate was washed with 75% acetone. The DNA was dissolved in 10 μl of formamide/phosphate buffer containing ^{125}I-labeled rRNA, covered with mineral oil, incubated at 100°C for 10 min, then hybridized at 44°C for various times. RNase-resistant hybrid was measured. ○, Sonicated DNA, no agarose; □, sonicated DNA, 0.6% agarose; ●, sonicated DNA, 1.5% agarose; ■, unsheared DNA, 1.5% agarose. Chain length: sonicated DNA, 400–600 nucleotides; unsheared DNA, 10,000–15,000 nucleotides. C_0t, initial concentration of DNA in mol of nucleotides per liter multiplied by time in sec. Percent hybridization was corrected for that fraction of the RNA that did not hybridize at high C_0t values (≈40%).

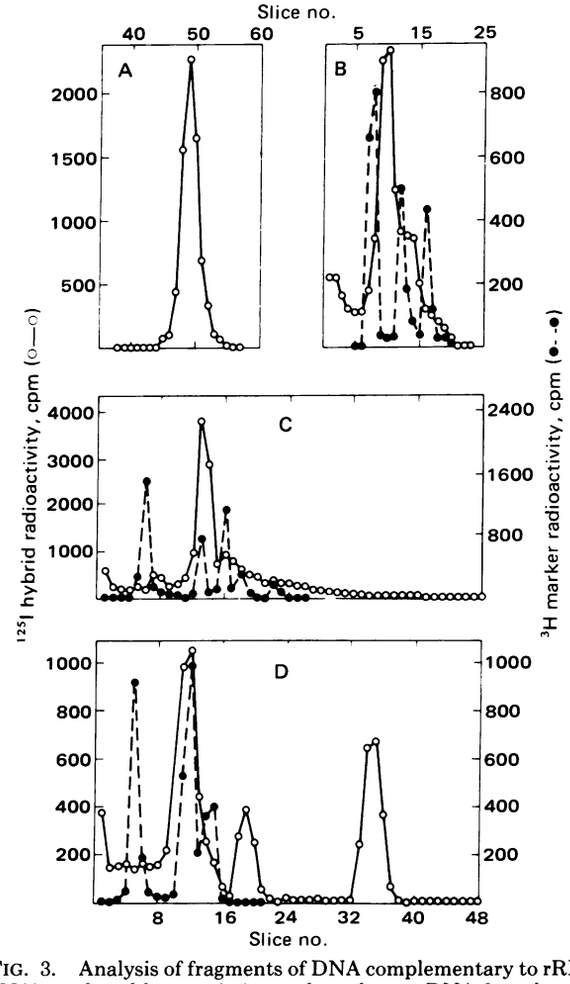

FIG. 3. Analysis of fragments of DNA complementary to rRNA (rDNA) produced by restriction endonucleases. DNA from human NC37 cells infected with simian sarcoma virus was digested with *Hin*dIII and 10 mg was fractionated on a giant agarose cylinder (unpublished data). The gel was sectioned into 5-mm slices, and rDNA sequences were located by our procedure on 0.2% aliquots of each slice. Slices containing rDNA were dissolved in NaI and the DNA was recovered by binding to glass. [^{3}H]DNA from bacteriophage λ was added and new fragments, produced by restriction enzymes on the initial $rDNA_{HindIII}$ fragment, were resolved by analytical electrophoresis through slabs of 1% agarose. Gels were sliced and rDNA sequences were located. (A) No secondary digest; (B) redigested with *Hin*dIII; (C) redigested with *Eco*RI; (D) redigested with *Bam*HI. ○, Hybridization to ^{125}I-labeled rRNA; ●, [^{3}H]DNA marker.

Table 1. Sample calculations from results of Fig. 3D: *Bam*HI map of human 28S rDNA

DNA fragment length, bp	RNA cpm hybridized	cpm/bp	Relative cpm hybridized	Sequence length, bp	
				Codogenic DNA	Noncodogenic DNA
6930	2673	0.38	1.45	2465	4465
3540	1037	0.29	0.58	986	2554
1700*	1835	1.04	1.00	1700	0
4040	0	0	0	0	4040
16,210	—	—	—	5151	11,059

Radioactivity under each peak was summed, then divided by the number of base pairs (bp) of DNA in the relevant fragment. The peak with the highest cpm/bp (1700 bp) was set to a relative cpm hybridized value of 1.00 and other peaks were normalized to it. The DNA fragment length times this value yielded the codogenic sequence length. The noncodogenic sequence length was obtained by subtraction. The codogenic sequence length of the three *Bam*HI fragments is 5151 bp; thus the spacer is 11,059 bp (16,210 minus 5151). The noncodogenic sequence lengths of the three *Bam*HI fragments is 7019 bp; thus there must be 4040 bp of pure spacer fragments not detected.

* Determined separately on gels of 3% agarose.

concentrations of formamide, DNA reannealing does not compete with RNA·DNA hybridization (16, 17). With appropriate ionic modifications the technique was readily adapted to DNA·DNA hybridizations, assayed with nucleases or hydroxyapatite (unpublished data).

Coupled Preparative and Analytical Techniques. The two methods described above can be combined to advantage for restriction enzyme mapping of DNA fragments and for gene purification. Fig. 3 presents a secondary analysis of a DNA fragment produced by treating human DNA with *Hin*dIII and containing sequences complementary to 28S rRNA. This $11 \cdot 10^6$-dalton fragment was located in a preparative agarose gel (D. Strayer, personal communication), by acetone precipitation, and freed of agarose by the glass-binding technique. It was mixed with [^{3}H]DNA from bacteriophage λ. Then aliquots were treated with no enzyme, with *Hin*dIII, with *Eco*RI, or with *Bam*HI. Each sample was run out on a slab gel, and slices were analyzed for sequences complementary to 28S rRNA. The 16,210-base-pair original fragment (Fig. 3A) is a limit digestion product of *Hin*dIII (Fig. 3B). Subsequent digestion by *Eco*RI leaves a fragment of about 7000 base pairs, close to the 7300 base pairs expected from Arnheim and Southern's results (18). *Bam*HI leaves three fragments containing sequences complementary to 28S rRNA (Fig. 3D). The ratio of hybridization signal to DNA fragment molecular weight (cpm/base pair) allows the classification of the smallest fragment as internal and the larger two as terminal (Table 1). There must also exist one or more fragments containing only spacer sequences (Table 1).

DISCUSSION

Most techniques available for separating DNA from agarose have limitations (13). Elution from the gel electrophoretically, by passive diffusion, or by "freeze-squeeze" (3–8, 12) fails to quantitatively recover high molecular weight DNA and yields material contaminated with agarose (6). Some methods have been devised that use agarose dissolution in chaiotropic salts such as sodium perchlorate (9) or potassium iodide (10). Most commonly, DNA is then bound to hydroxyapatite (11). We have been unable to prepare undegraded high molecular weight DNA with this method and have experienced difficulties digesting recovered DNA with some restriction enzymes. Furthermore, recovery of the DNA in phosphate buffer presents annoying problems. Occasionally, centrifugation to equilibrium is used as a second step (10). This method is quite satisfactory for recovering undegraded high molecular weight DNA, but is laborious and is unsuitable for recovery of low molecular weight DNA.

Binding DNA to glass, after dissolving the agarose in NaI, is a satisfactory means of removing DNA from agarose. It is rapid, convenient, and nearly quantitative, and yields DNA of high purity. The binding of DNA to glass is complete (over 99%), and elution of the DNA is about 90% efficient with 10 μl of buffer per mg of glass–DNA complex. With small volumes (1–10 ml), binding is complete in minutes; with larger volumes (e.g., 100 ml), an overnight binding is usually required. The capacity of glass powder exceeds 1 mg of DNA bound per g of glass. Thus, DNA can be recovered at concentrations of 100 μg/ml, independent of the concentration of DNA in the gel. DNA is recovered in any convenient buffer.

The glass-binding method is particularly suitable for recovering DNA from agarose for further analysis or purification. In spite of its convenience for preparative purposes, however, it is not sufficiently convenient for monitoring DNA sequences in sliced agarose gels in analytical experiments. For analytical purposes two techniques are now available, the Southern transfer system (19) and precipitation from acetone (our technique). Southern transfers are qualitative and comparative, whereas our technique is quantitative. Quantitation is attained because hybridization is carried out in liquid with an excess of unlabeled DNA fragment under conditions where DNA fragment reannealing is eliminated, by using RNA probes (16, 17), or where it is not a serious theoretical problem (e.g., by using DNA probes).

Quantitation permits the elucidation of several parameters. At high C_0t, the proportion of the labeled probe complementary to each fragment is obtained, while at lower C_0t the relative abundance of each fragment is measured. The 16,210-base-pair fragment of the human rDNA cistron left by *Hin*dIII contains all of the sequences codogenic for 28S rRNA and *Bam*HI cuts the codogenic region twice, leaving three fragments complementary to the RNA (Fig. 3D). The ratio of radioactivity hybridized to DNA fragment length (cpm/base pair) shows that the smallest fragment is internal. The two larger fragments hybridize less RNA per unit DNA length, so they contain noncodogenic regions in addition to portions that are complementary to the RNA. The spacer on each side of the 28S cistron was precisely evaluated with respect to the positions of *Bam*HI sites by simply subtracting the codogenic 28S base pairs from the total fragment length (Table 1). Thus, the 3540-base-pair fragment extends about 2500 base pairs into the short spacer between the 28S and 18S cistrons and the 6930-base-pair fragment extends 4465 base pairs into the large spacer on the other side of the 28S gene. Of the 16,210-base-pair *Hin*dIII fragment, about 12,000 base pairs are accounted for by *Bam*HI fragments carrying 28S sequences. Thus, about 4000 base pairs of pure spacer fragment(s) are also produced. These are not detected by the 28S rRNA probe.

Hybridization with excess DNA obviously circumvents the detection of fragments complementary to impurities in the

probe. The technique is compatible with analyses of the properties of the hybrid formed between each DNA fragment and the radioactive probe, especially its thermal stability. In principle, perfectly complementary sequences can be distinguished from related sequences. Finally, the capacity for including internal markers provides a monitor for the extent of nuclease digestion, yields precise molecular weight estimates, and allows a rough estimate of sequence purity in a hybridization peak (from band-width parameters).

The length of time required for a particular hybridization can be calculated within acceptably narrow limits, given the amount of DNA run out on an agarose gel, the fraction of each slice used, the approximate width of the bands, and the copy number of the sequence of interest. Consequently, single-copy sequences have been amenable to analysis with both RNA and DNA probes (unpublished data).

Southern transfers and our technique have complementary attributes. Southern transfers are the ultimate in comparative convenience and provide a satisfying visual display of the results. They are particularly suitable for surveys to indicate the situations that might be profitably studied in quantitative detail by our technique. Clearly, the combination of these two techniques with preparative removal of DNA from agarose and preparative gel electrophoresis provides a reasonable starting point for dismantling complex genomes.

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